Inflammation, Vol. 27, No. 4, August 2003 (© 2003)

Differential Activation of Signal Transduction Pathways Mediating Oxidative Burst by Chicken Heterophils in Response to Stimulation with Lipopolysaccharide and Lipoteichoic Acid

Morgan B. Farnell,[1] Haiqi He,[2] and Michael H. Kogut[2,3]

Abstract—Toll-like receptors (TLRs) have been previously shown to mediate oxidative burst in chicken heterophils. This study was conducted to begin to map the molecular pathways that regulate TLR-mediated oxidative burst. Peripheral blood heterophils from neonatal chicks were isolated and exposed to known inhibitors of signal transduction pathways for either 20 min (genistein, verapamil, or chelerythrine) or 120 min (pertussis toxin) at 39°C. The cells were then stimulated for 30 min at 39°C with *Salmonella enteritidis* lipopolysaccharide (LPS) or *Staphylococcus aureus* lipoteichoic acid (LTA). The heterophil oxidative burst was then quantitated by luminol-dependent chemiluminescence (LDCL). Genistein (a tyrosine kinase inhibitor), verapamil (a calcium channel blocker), chelerythrine (a protein kinase C inhibitor), and pertussis toxin (a G-protein inhibitor) significantly reduced LPS-stimulated oxidative burst in chicken heterophils by 34, 50, 63, and 51%, respectively. Although genistein had a statistically significant effect on reducing LPS-stimulated LDCL biologically it seems to play only a minor role within the oxidative burst pathway. Heterophils stimulated with the gram-positive TLR agonist, LTA, activated a different signal transduction pathway since chelerythrine was the only inhibitor that significantly reduced (72%) LTA-stimulated oxidative burst. These findings demonstrate that distinct signal transduction pathways differentially regulate the stimulation of oxidative burst in avian heterophils. Pertussis toxin-sensitive, protein kinase C-dependent, Ca^{++}-dependent G proteins appear to regulate oxidative burst of avian heterophils stimulated with gram-negative agonist LPS; whereas, a protein kinase C-dependent signal transduction pathway plays the major role activating the oxidative burst of avian heterophils stimulated with gram-positive agonists. The distinct differences in the response of heterophils to these two agonists illustrate the specificity of TLRs to pathogen-associated molecular patterns (PAMP)s.

KEY WORDS: signal transduction; heterophils; oxidative burst; chicken; Toll-like receptors.

INTRODUCTION

Heterophils, the avian equivalent to the mammalian neutrophil, are essential cellular components of the avian innate immune system (1, 2). These granulocytic phagocytes kill pathogens by the release of toxic oxygen metabolites (oxidative burst) and the release of lytic enzymes and antimicrobial peptides (degranulation) (3).

[1]Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, Texas.

[2]USDA-ARS, Southern Plains Agricultural Research Center, College Station, Texas.

[3]To whom correspondence should be addressed at USDA-ARS, 2881 F&B Road, College Station, Texas 77845. E-mail: kogut@ffsru.tamu.edu

Abbreviations: HBSS, Hank's balanced salt solution; LDCL, luminol dependent chemiluminescence; LPS, lipopolysaccharide; LTA, lipoteichoic acid; PAMP, pathogen associated molecular pattern; PBS, phosphate buffered saline; PMN, polymorphonuclear; PRR, pattern recognition receptor; SCWL, single-comb white leghorn; TLR, Toll-like receptor.

0360-3997/03/0800-0225/0

Heterophils, the second most numerous cell in the peripheral blood, can respond to invading microorganisms within 30 min of infection and are more efficient at phagocytizing and killing than macrophages (4, 5).

Mammalian neutrophils have been shown to recognize pathogens by pattern recognition receptors (PRRs) that interact via pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), found in the cell wall of gram-negative bacteria and lipoteichoic acid (LTA), found in the cell wall of gram-positive bacteria (6–10). One group of PRRs, known as Toll-like receptors (TLRs), has been shown to be an important component of the innate immune system in several species, such as *Drosophila*, humans, plants, and chickens (11–14). Phagocytes utilize TLRs to identify specific pathogen associated molecular patterns and ultimately direct the adaptive immune system through antigen presentation to lymphocytes (8). TLRs have also been shown to mediate oxidative burst in mammalian neutrophils (15, 16). We have recently shown that avian heterophils also possess TLRs that mediate oxidative burst (14).

While TLR signal transduction pathways have been identified in mammalian leukocytes, a signal transduction pathway for TLR-mediated oxidative burst has not been identified in avian heterophils. The objective of the present study was to evaluate the association of signal transduction factors with the enhancement of oxidative burst of avian heterophils stimulated with agonists from gram-negative (LPS) and gram-positive (LTA) bacteria. Four prominent signal transduction factors or second messengers involved in neutrophil activation include protein kinase C, tyrosine kinases, intracellular Ca^{++} ions, and G-proteins (3). In these experiments, we compared the effects of genistein (tyrosine kinase inhibitor), verapamil (calcium channel blocker), chelerythrine (protein kinase C inhibitor), and pertussis toxin (G-protein inhibitor) on the ability of LPS and LTA to stimulate oxidative burst *in vitro*.

MATERIALS AND METHODS

Experimental Animals

Single-comb white Leghorn male chickens (Hy-Line W-36) were obtained on the day-of-hatch from a commercial hatchery (Hy-Line International, Bryan, TX) and placed in floor pens with supplemental heat. Chicks were provided water and a balanced, unmedicated, corn-soybean ration *ad libitum* that met or exceeded the National Research Council guidelines for chicken nutrition (17).

Isolation of Heterophils

Blood was collected by decapitation and EDTA was used as an anticoagulant. Blood was pooled from 50–100 neonates and peripheral heterophils isolated as previously described (10). Briefly, blood was mixed with 1% methylcellulose (Sigma, St. Louis, MO) in a 1.5:1 ratio and centrifuged at $25 \times g$ for 15 min. The supernatant was removed and resuspended in Hanks balanced salt solution without calcium and magnesium at a 1:1 ratio. The suspension was then layered over a 1.077/1.119 Ficoll-Hypaque gradient (Sigma, St. Louis, MO) and centrifuged at $250 \times g$ for 60 min. Following a RPMI wash, the cells were quantitated using a Neubauer hemacytometer and the concentration was adjusted to 4×10^6 heterophils/mL. Cell viability was routinely $>95\%$ as determined by trypan blue exclusion. The purity of the heterophil suspensions was assessed by microscopic examination of Diff-Quick-stained cytospin (Shandon Scientific, Pittsburgh, PA) smears. Heterophil preparations obtained by this method were typically $>95\%$ pure.

Agonists

LPS and LTA (Sigma, St. Louis, MO) were dissolved in DMSO and diluted in RPMI to stock concentrations of 1 mg/mL and stored at 20°C until used. The final concentration of DMSO in the experiments was less than 0.5%. The effective concentration of agonist was previously determined by a dose response (14).

Polyclonal Antibodies to CD14, TLR2, and TLR4

Goat polyclonal antibodies raised against peptides near the amino terminus of rat CD14, the carboxy terminus of human TLR2 (TLR2C), and the carboxy terminus of human TLR4 were purchased from Santa Cruz Biotechnology Lab (Santa Cruz, CA). Preimmune isotype matched goat IgG was used as a negative control (Santa Cruz Biotechnology Lab, Santa Cruz, CA). Antibodies were used at a concentration of 2.0 μg/mL as suggested by the manufacturer and verified by dose response. The heterophils and antibodies were incubated, prior to stimulation with the agonists; at 39°C for one hour in 15 mL screw capped polypropylene conical tubes on a rocker plate.

Inhibitors

Genistein, verapamil, chelerythrine, and pertussis toxin were obtained from Sigma Chemical Co. (St. Louis,

Table 1. Signal Transduction Inhibitors Used in These Studies

Inhibitors	Function
Genistein	Inhibits tyrosine kinase activity
Verapamil	Ca^{++} channel blocker
Chelerythrine	Inhibits protein kinase C activity
Pertussis toxin	Inhibits regulatory action of G-proteins

MO). With the exception of pertussis toxin, all of the inhibitors were dissolved in DMSO and stock solutions of genistein (100 μM), verapamil (1000 μM), chelerythrine (100 μM) were stored at 4°C until used. A stock solution of pertussis toxin (10 μg/mL) was prepared in PBS and stored at 4°C until used. Working concentrations of the inhibitors were prepared in RPMI 1640 tissue culture medium (Sigma, St. Louis, MO) from the stock concentrations. The final concentration of DMSO in the experiments was less than 0.5%. The function of each inhibitor is described in Table 1. The inhibitors genistein, verapamil, and chelerythrine were used at a concentration of 100 μM. Pertussis toxin was used at a concentration of 1000 μg/mL (Sigma, St. Louis, MO). With the exception of pertussis toxin, the heterophils and inhibitors were incubated, prior to stimulation with the agonists; at 39°C for 20 min in 15 mL screw capped polypropylene conical tubes on a rocker plate. Cells treated with pertussis toxin were incubated for 2 h prior to stimulation.

Luminol-Dependent Chemiluminescence (LDCL) Assay

Oxidative burst of heterophils was measured by use of a luminol dependent chemiluminescence assay adapted from a previously described procedure (18). Briefly, heterophils (8×10^5) and luminol (0.1 M, Sigma, St. Louis, MO) in 0.5 mL of RPMI were placed in polypropylene scintillation vials and stimulated with LPS or LTA for 30 min. Luminol-dependent chemiluminescence was measured in a 1219 RackBeta liquid scintillation counter (LKB Wallac, Turku, Finland), using the tritium channel and the incoincidence mode. All samples were assayed in replicates of five tubes per treatment.

Experimental Design

To determine if CD14 and TLRs mediate heterophil oxidative burst, cells were incubated for 60 min at 39°C in a 15 mL conical tube with the appropriate amount of antibody or left untreated. The samples were incubated in the dark for 30 min with the agonist at room temperature prior to LDCL measurement. Each trial was repeated in three identical experiments.

To determine which signal transduction pathways are involved in TLR-mediated oxidative burst, cells were incubated for 20 or 120 min at 39°C in a 15 mL conical tube with the appropriate amount of inhibitor or left untreated. The samples were incubated in the dark for 30 min with the agonist at room temperature prior to LDCL measurement. Each trial was repeated in three identical experiments.

Statistical Analysis

Data was pooled for each group and compared to a stimulated control using a paired T test for the LTA and LPS treatments from Sigma Stat 2.0 statistical software (Jandel Corporation, Chicago, IL).

RESULTS

Neutralization of Oxidative Burst by TLR and CD14 Polyclonal Antibodies

Heterophils were preincubated with CD14 Ab, TLR2C Ab, TLR4 Ab, isotype control IgG, or RPMI only (Fig. 1). All groups were then stimulated with 10 μg/mL of LPS or LTA and LCDL was measured. Isotype matched preimmune goat IgG (2.0 μg/mL) nonspecifically inhibited heterophil oxidative burst by 36.1% when stimulated with 10 μg/mL of LPS. Heterophils treated with goat anti-human CD14 antibody had no significant effect on oxidative burst when stimulated with LPS when compared to the IgG control treated cells. Treatment of heterophils with the goat anti-human TLR2 and TLR4 antibodies significantly ($p < 0.001$) decreased oxidative burst when stimulated with LPS when compared to the IgG control (Fig. 1).

The isotype matched goat IgG had no nonspecific inhibitory activity on heterophil oxidative burst when stimulated with 10 μg/mL of LTA. Goat anti-human CD14 and TLR2 antibodies significantly ($p < 0.001$) decreased oxidative burst by 36.6 and 35.2%, respectively, when the heterophils were stimulated with LTA. Anti-human TLR4 antibody had no effect on oxidative burst in heterophils stimulated with LTA (Fig. 1).

Neutralization of Oxidative Burst by Signal Transduction Inhibitors

There were interesting differences in the signaling pathways of heterophils stimulated with LTA or LPS.

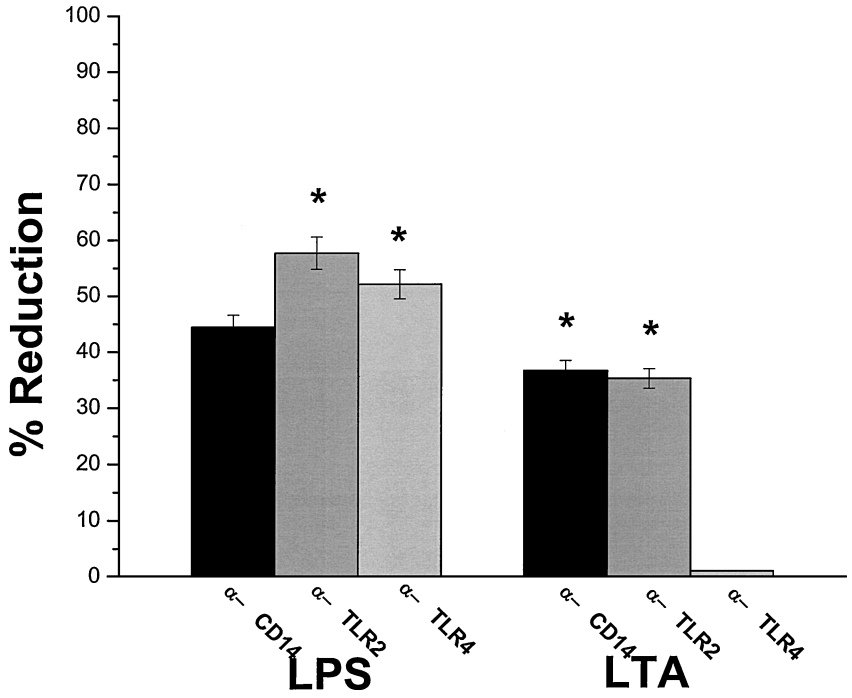

Fig. 1. The effect of CD14 and TLR blocking antibodies on the luminol dependent chemiluminescence (LCDL) of avian heterophils stimulated with *Salmonella enteritidis* lipopolysaccharide (LPS) or *Staphylococcus aureus* lipoteichoic acid (LTA). Heterophils were incubated with antibody for 1 h at 39°C. Heterophils were then incubated with LPS or LTA for 30 min in luminol and LCDL was measured as an indicator of oxidative burst. Data was pooled from three repetitions and presented as the percent reduction in oxidative burst as compared to control heterophils. Significant differences were determined using a paired T test (* represents $p < 0.014$ compared to control heterophils).

Chelerythrine was the only inhibitor that inhibited LTA-stimulated oxidative burst (72%, Fig. 2). However, all four inhibitors, genistein, verapamil, chelerythrine, and pertussis toxin inhibited LPS-stimulated oxidative burst by 34, 50, 63, and 51%, respectively (Fig. 2). These data indicate that only protein kinase C is involved in LTA-stimulated oxidative burst, while tyrosine kinase, calcium channels, protein kinase C, and G proteins are involved in LPS-stimulated oxidative burst (Fig. 2).

DISCUSSION

Bacterial infections in meat producing animals are usually controlled with the prophylactic use of antibiotics, which are also used at subtheraputic levels as growth promoters. Unfortunately, bacteria have acquired resistance to these antibiotics, and instead of preventing bacterial infections and cases of food-borne illness the consumer is infected with antibiotic resistant strains that are even more difficult to treat (19–21). The subtheraputic and prophylactic use of antibiotics in food animals is being phased out as a production practice, and new alternative treatments will be needed to treat disease. One possibility would be to manipulate the appropriate signal transduction pathways to stimulate an immune response that would prevent or help clear an infection (22, 23).

Intracellular signal transduction is a series of elaborate biochemical pathways regulated by multiple enzyme systems and secondary messengers (3). Several of these signal transduction enzymes and secondary messengers have been shown to be involved in the functional stimulation of mammalian neutrophils and avian heterophils including tyrosine kinase, intracellular Ca^{++} ions, protein kinase C, and G-proteins (3, 24–29).

Genistein (a tyrosine kinase inhibitor), verapamil (a calcium channel blocker), chelerythrine (a protein kinase C inhibitor), and pertussis toxin (a G-protein inhibitor) were used in the present experiment to determine which molecular pathways are involved in regulating

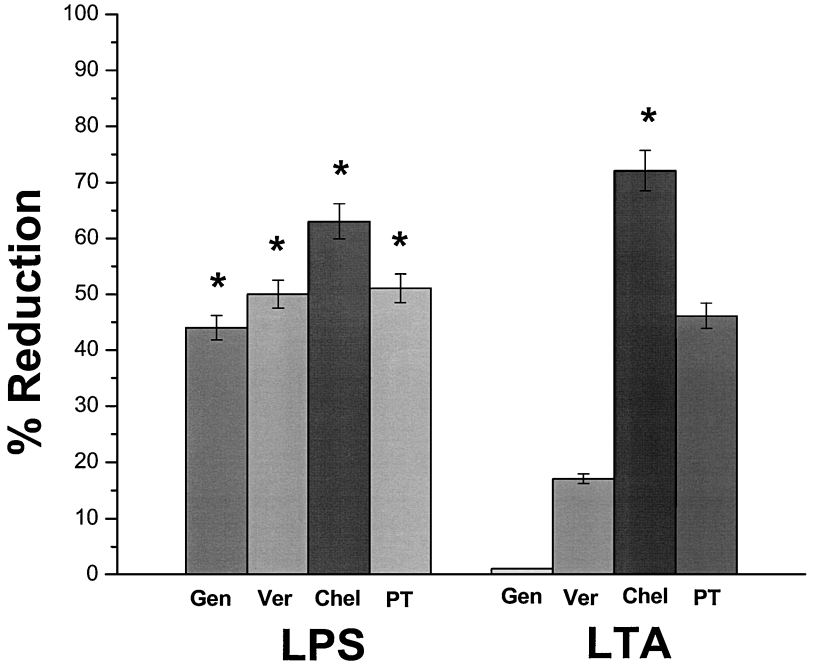

Fig. 2. The effect of signal transduction inhibitors on *Salmonella enteritidis* lipopolysaccharide (LPS) or *Staphylococcus aureus* lipoteichoic acid (LTA) stimulated luminol-dependent chemiluminescence of neonatal chicken heterophils. Heterophils were incubated with inhibitor for 20 (genistein, verapamil, and chelerythrine) to 120 (pertussis toxin) min at 39°C. Heterophils were then incubated with LPS or LTA for 30 min in luminol and LCDL was measured as an indicator of oxidative burst. Data was pooled from three repetitions and presented as the percent reduction in oxidative burst as compared to control heterophils. Significant differences were determined using a paired T test (* represents $p < 0.026$ compared to control heterophils).

oxidative burst in avian heterophils. These inhibitors have been previously used in combination with stimulants such as opsonized zymosan, opsonized SE, and phorbol myristate acetate in avian heterophils. However, the use of TLR agonists such as LPS and LTA to elucidate the signal transduction pathways involved in the TLR-mediated oxidative burst in chicken heterophils has not been examined (3, 29).

We previously demonstrated that LPS and LTA are potent inducers of oxidative burst in avian heterophils and that these events are mediated by TLRs (14), we have confirmed these finding here (Fig. 1). Our research demonstrated that TLR2 and TLR4 mediate LPS-stimulated oxidative burst in heterophils, while CD14 and TLR2 mediate LTA-stimulated oxidative burst in heterophils. Knowledge of the biochemical mechanisms of the functional activity of avian heterophils is required to understand the regulation of the inflammatory response (3). Studies conducted here begin to delineate the signal transduction pathways involved in TLR-mediated oxidative burst in avian heterophils.

In our studies, treatment of heterophils with genistein significantly inhibited LPS-stimulated oxidative burst, but it is doubtful that the reduction was biologically significant because of the low percent reduction observed. Similarly, the inhibitory effect of genistein on heterophils stimulated with LTA was negligible; however, genistein was effectively used in previous papers to inhibit the oxidative burst of avian and bovine granulocytes (3, 24). These results are expected because of the fact that opsonized zymosan and opsonized SE were used in the previous experiments versus nonopsonized agonists that we used. The differences in responses illustrate the difference between Fc receptor and TLR-mediated oxidative burst in chicken heterophils.

We found that verapamil inhibited LPS-stimulated oxidative burst, but not LTA-stimulated oxidative burst. Kogut *et al.* (3) and Yu and Czuprynski (24) reported similar results using Fc receptor agonists. Ortiz-Carranza and Czuprynski (25) used *Pasteurella haemolytica* leukotoxin, a TLR agonist, to stimulate bovine neutrophils.

The *haemolytica*-stimulated neutrophils responded with a significantly reduced oxidative burst when inhibited with verapamil. These reports substantiate the importance of calcium ions for oxidative burst in polymorphonuclear (PMN) leukocytes. However, they do not explain why verapamil did not inhibit LTA-stimulated oxidative in avian heterophils. TLRs have been shown to react differentially to PRRs in mammalian phagocytes. TLR 2, for instance, has been reported to have a high affinity for lipoteichoic acid while TLR 4 is reported to have a high affinity for lipopolysaccharide in mammalian cells (11). Similarly, the data suggests that LPS is binding a different TLR than the LTA and initiating a different signal transduction pathway.

Among the inhibitors used, chelerythrine was the only inhibitor that inhibited oxidative burst in heterophils stimulated by LPS and LTA. This indicates that protein kinase C is involved in both pathways. Although the combinations of inhibitors and agonists that we used have not been previously published, previous papers have demonstrated the importance of protein kinase C to neutrophil oxidative burst, chemotaxis, adherence, and degranulation (3, 24, 26). It is interesting that protein kinase C plays such a large role in LTA-stimulated oxidative burst while calcium ions, which usually induces protein kinase C migration and binding to the plasma membrane to be later activated by DAG, does not seem to be involved.

Pertussis toxin was shown to inhibit LPS-stimulated oxidative burst that suggest that G proteins are involved in heterophil oxidative burst. However, pertussis toxin had no effect on LTA-stimulated oxidative burst. The importance of G proteins in neutrophil/heterophil activation has been previously demonstrated in chicken, bovine, and human phagocytes (3, 24, 27). They have been shown to be involved in oxidative burst, phagocytosis, degranulation, and chemotaxis pathways.

In conclusion, this is the first report on the signal transduction pathways involved in TLR-mediated oxidative burst in avian heterophils. On the basis of the data reported here, oxidative burst stimulated by LPS is regulated by a pertussis toxin sensitive, protein kinase C-dependent, Ca^{++} dependent, G-protein. LTA-stimulated heterophils, however, are not regulated by tyrosine kinases, Ca^{++} ions, or G proteins, but instead are regulated by protein kinase C. These data demonstrate the ability of TLRs to recognize and differentiate between gram-positive and gram-negative bacteria. We plan to further our studies by looking at more specific intermediary enzymes and secondary messengers involved in oxidative burst.

REFERENCES

1. Harmon, B. G. 1998. Avian heterophils in inflammation and disease resistance. *Poult. Sci.* **77:**972–977.
2. Kogut, M. H., G. I. Tellez, E. D. McGruder, B. M. Hargis, J. D. Williams, D. E. Corrier, and J. R. DeLoach. 1994. Heterophils are decisive components in the early responses of chickens to *Salmonella enteritidis* infections. *Microb. Pathol.* **16:**141–151.
3. Kogut, M. H., K. J. Genovese, and V. K. Lowry. 2001. Differential activation of signal transduction pathways mediating phagocytosis, oxidative burst, and degranulation by chicken heterophils in response to stimulation with opsonized *Salmonella enteritidis*. *Inflammation* **25**(1):7–15.
4. Kogut, M. H., E. D. McGruder, B. M. Hargis, D. E. Corrier, and J. R. DeLoach. 1995. *In vivo* activation of heterophil function in chickens following injection with *Salmonella enteritidis*—immune lymphokines. *J. Leukoc. Biol.* **57:**56–62.
5. Stabler, J. G., T. McCormick, K. Powell, and M. H. Kogut. 1994. Avian heterophils and monocytes: Phagocytic and bactericidal activities against *Salmonella enteritidis*. *Vet. Microbiol.* **38:**293–305.
6. Medzhitov, R. and C. A. Janeway Jr. 1997. Innate immunity: The virtues of a nonclonal system of recognition. *Cell* **91:**295–298.
7. Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid- induced cell activation is mediated by Toll-like receptor 2. *J. Biol. Chem.* **274**(18):17406–17409.
8. Medzhitov, R. and C. A. Janeway Jr. 1997. Innate immunity: Impact on the adaptive immune response. *Curr. Opin. Immunol.* **9:**4–9.
9. Janeway, C. A., Jr. and R. Medzhitov. 1999. Innate immunity: Lipoproteins take their Toll on the host. *Curr. Biol.* **9:**R879–R882.
10. Medzhitov, R. and C. A. Janeway Jr. 2000. The Toll receptor family and microbial recognition. *Trends Microbiol.* **8**(10):452–456.
11. Akira, S., K. Takeda., and T. Kaisho. 2001. Toll-like receptors: Critical proteins linking innate and acquired immunity. *Nature Immunol.* **2**(8):675–680.
12. Boyd, Y., M. Goodchild., S. Morroll, and N. Bumstead. 2001. Mapping of the chicken and mouse genes for Toll-like receptor 2 (TLR2) to an evolutionarily conserved chromosomal segment. *Immunogenetics* **52:**294–298.
13. Fukui, A., N. Inoue, M. Matsumoto, M. Nomura, K. Yamada, Y. Matsuda, K. Toyoshima, and T. Seya. 2001. Molecular cloning and functional characterization of chicken Toll-like receptors. *J. Biol. Chem.* **276**(50):47143–47149.
14. Farnell, M. B., T. L. Crippen, H. He, C. L. Swaggerty, and M. H. Kogut. 2003. Oxidative burst mediated by Toll-like receptors (TLR) and CD14 on avian heterophils stimulated with bacterial Toll agonists. *Dev. Comp. Immunol.* **27**(5):423–429.
15. Aliprantis, A. O., D. S. Weiss, and A. Zychlinsky. 2001. Toll-like receptor-2 transduces signals for NF-kappa B activation, apoptosis and reactive oxygen species production. *J. Endotoxin Res.* **7**(4):287–291.
16. Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski, and A. Zychlinsky. 1999. Cell activation and apoptosis by bacterial lipoproteins through Toll-like receptor-2. *Science* **285**(5428):736–739.
17. National Research Council. 1994. *Nutrient Requirements of Poultry*, 9th edn. National Academy Press, Washington, DC.
18. Merrill, G. A., R. Bretthauer, J. Wright-Hicks, and R. C. Allen. 1996. Oxygenation activities of chicken polymorphonuclear leukocytes

investigated by selective chemiluminigenic probes. *Lab. Anim. Sci.* **46**(5):530–538.
19. Raloff, J. 1998. Livestock's role in antibiotic resistance. *Sci. News* **154**(3):39.
20. Ferber, D. 2002. Livestock feed ban preserves drugs' power. *Science* **295**(5552):27–28.
21. Lowenthal, J. W., B. Lambrecht, T. P. van den Berg, M. E. Andrew, A. D. G. Strom, and A. G. D. Bean. 2000. Avian cytokines—The natural approach to therapeutics. *Dev. Comp. Immunol.* **24:**355–365.
22. Lodish, H., A. Berk, S. L. Zipursky, D. Matsudaira, D. Baltimore, and J. E. Darnell. 2000. Cell-to-cell signaling: Hormones and receptors. In: *Molecular Cell Biology*, 4th edn., Ch. 20. Freeman, New York, pp. 848–909.
23. Janeway, C. A., P. Travers, M. Walport, and M. Shlomchik. 2001. Signaling through immune system receptors. In: *Immunobiology: The Immune System in Health and Disease*, 5th edn., Ch. 6. Garland, New York, pp. 187–220.
24. Yu, P. W. and C. J. Czuprynski. 1996. Regulation of luminol-dependent chemiluminescence and degranulation by bovine neutrophils stimulated with opsonized zymosan. *Vet. Immunol. Immunopathol.* **50**(1/2):29–42.
25. Ortiz-Carranza, O. and C. J. Czuprynski. 1992. Activation of bovine neutrophils by *Pasteurella haemolytica* leukotoxin is calcium dependent. *J. Leukoc. Biol.* **52**(5):558–564.
26. Laudanna, C., D. Mochly-Rosen, T. Liron, G. Constantin, and E. C. Butcher. 1998. Evidence of zeta protein C involvement in polymorphonuclear neutrophil integrin-dependent adhesion and chemotaxis. *J. Biol. Chem.* **273**(46):30306–30315.
27. Tilton, B., M. Andjelkovic, S. A. Didichenko, B. A. Hemmings, and M. Thelen. 1997. G-protein-coupled receptors and Fc gamma-receptors mediate activation of Akt/protein kinase B in human phagocytes. *J. Biol. Chem.* **272**(44):28096–28101.
28. Crockett-Torabi, E. and J. C. Fantone. 1990. Soluble and insoluble immune complexes activate human neutrophil NADPH oxidase by distinct Fc gamma receptor-specific mechanisms. *J. Immunol.* **145**(9):3026–3032.
29. Kogut, M., V. K. Lowry, and M. Farnell. 2002. Selective pharmacological inhibitors reveal the role of *Syk* tyrosine kinase, phospholipase C, phosphatidylinositol-3′-kinase, and p38 mitogen-activated protein kinase in Fc receptor-mediated signaling of chicken heterophil degranulation. *Int. Immunopharmacol.* **2**(7):963–973.